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Efeito do treinamento físico aeróbico sobre biomarcadores inflamatórios, densidade mitocondrial e capacidade de exercício em modelo animal que mimetiza menopausa

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EFEITO DO TREINAMENTO FÍSICO AERÓBICO SOBRE BIOMARCADORES INFLAMATÓRIOS, DENSIDADE MITOCONDRIAL E CAPACIDADE DE EXERCÍCIO EM MODELO ANIMAL QUE MIMETIZA MENOPAUSA

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RESUMO

Objetivo: Investigar o efeito do treinamento de endurance moderado e da reposição de estradiol na capacidade aeróbia do músculo esquelético, densidade mitocondrial, estado redox e biomarcadores inflamatórios de ratas ovariectomizadas.

Métodos: Ratas Wistar com doze semanas de idade foram distribuídas aleatoriamente em três grupos: Ratas ovariectomizadas não aerobicamente treinadas (OVX-NAT), Ratas ovariectomizadas com reposição estrogênica (OVX-ER) e Ratas ovariectomizadas aerobicamente treinadas (OVX-AT). O treinamento consistiu em corrida em esteira a 50 ~ 70% da velocidade máxima de corrida, uma hora por dia, 5 dias / semana durante 8 semanas. Todos os animais foram submetidos a um teste de esforço máximo em esteira antes e após o protocolo de treinamento aeróbio. Após a eutanásia, o músculo sóleo foi processado para avaliações histológicas e bioquímicas. Os principais desfechos avaliados foram capacidade aeróbia (consumo de oxigênio, eficiência mecânica, tempo, velocidade e distância alcançada em um teste de esforço máximo), densidade mitocondrial, estado redox [*superoxide dismutase activity* (SOD), *catalase activity* (CAT), *total antioxidant capacity* (FRAP), *thiobarbituric acid reactive substances* (TBARS)] e biomarcadores inflamatórios [interleucina 6 (IL-6), interleucina 10 (IL-10) e *tumor necrosis factor alpha* (TNF-α)].

Resultados: O grupo OVX-AT apresentou melhora da capacidade aeróbia e aumento da densidade mitocondrial do músculo sóleo enquanto a reposição de estradiol não afetou esses parâmetros. O treinamento de resistência também promoveu aumento nas concentrações de SOD, FRAP, e IL-10, enquanto o estradiol aumentou a concentração de CAT, e reduziu a concentração de IL-6.

Conclusões: O treinamento físico de resistência é mais eficaz do que a terapia de reposição de estradiol para controlar os aspectos do desequilíbrio redox e inflamatório, melhorando o conteúdo mitocondrial do músculo esquelético.

Palavras-chave: Ovariectomia, treinamento de resistência, estresse oxidativo, densidade mitocondrial, citocinas, músculo esquelético.

ABSTRACT

Aims: To investigate the effect of moderate endurance training and estradiol replacement in skeletal muscle aerobic capacity, mitochondrial density, redox status and inflammatory biomarkers of ovariectomized rats.

Main methods: Twelve weeks-old female Wistar rats were randomly assigned into three groups: Ovariectomized not aerobically trained rats (OVX-NAT), Ovariectomized rats with estrogen replacement (OVX-ER) and, Ovariectomized aerobically trained rats (OVX-AT). The training consisted of treadmill running at 50~70% of maximal running speed, one hour a day, 5 days/week during 8 weeks. All animals were submitted to a maximal effort treadmill test before and after the aerobic training protocol. After euthanasia, the soleus muscle was processed for histological e biochemical evaluations.
The main outcomes measures were aerobic capacity (oxygen consumption, mechanical efficiency, time, speed and distance reached in a maximal exercise test), mitochondrial density, redox status [superoxide dismutase activity (SOD), catalase activity (CAT), total antioxidant capacity (FRAP), thiobarbituric acid reactive substances (TBARS)] and inflammatory biomarkers [interleukin 6 (IL-6), interleukin 10 (IL-10), tumor necrosis factor alpha (TNF-α)].

Key findings: The OVX-AT presented improved aerobic capacity and increased soleus muscle mitochondrial density while estradiol replacement had no effect on these parameters. Endurance trained also promoted an increase in SOD, FRAP and IL-10 levels, while estradiol increased CAT and reduced IL-6 levels.

Conclusion: Endurance exercise training was more effective than estradiol replacement therapy to control aspects of the redox and inflammatory imbalance improving the skeletal muscle mitochondrial content.

Keywords: Ovariectomy, endurance training, oxidative stress, mitochondria density, cytokines, skeletal muscle.

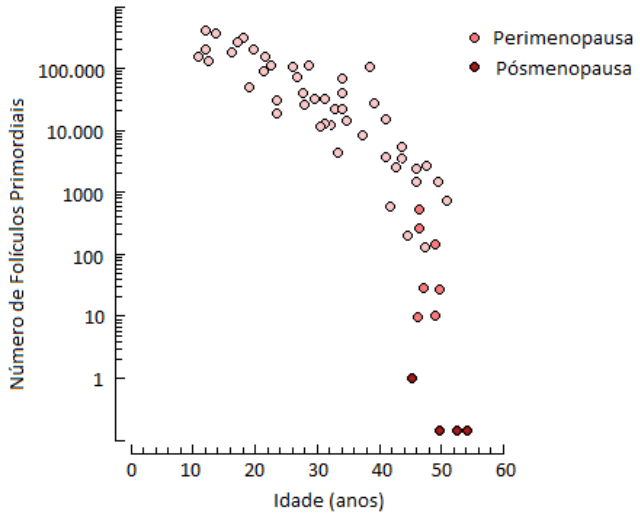
SUMÁRIO

1. INTRODUÇÃO

A menopausa é a cessação dos ciclos menstruais que ocorre em função do esgotamento dos folículos ovarianos. É diagnosticada após a ausência do ciclo menstrual por um tempo mínimo de 12 meses e ocorre naturalmente em média entre os 48 e 52 anos de idade. No entanto, a menopausa também pode acontecer precocemente, antes dos 45 anos, ou prematuramente, antes dos 40 anos, por causas espontâneas, como na falência ovariana prematura, ou induzidas, quando secundária à remoção cirúrgica dos ovários ou à falência ovariana iatrogênica (DAVIS *et al.*, 2015; SHUSTER *et al.*, 2010)

A menopausa caracteriza-se pela acentuada redução dos hormônios pelos folículos produzidos, tais como o estrogênio e a progesterona (DAVIS *et al.*, 2015). Dado o grande número de folículos primordiais com que as mulheres atingem a puberdade, cerca de quatrocentos mil, e dos quais 99,9% sofrem atresia, a razão pela qual os folículos ovarianos se esgotam nesta determinada idade ainda não foi estabelecida (RUTH *et al.*, 2016; TOWNSON; COMBELLES, 2012). Na figura 1 observa-se que o número de folículos ovarianos reduz em função da idade da mulher, e que esta redução é acentuada no período perimenopausa (BURGER, 2006).

Figura 1. Número de folículos ovarianos reduz com a idade



Adaptado de Burguer *et al*, 2006.

Embora a menopausa seja um processo natural, com a deficiência dos hormônios ovarianos no organismo, principalmente do estrogênio, ocorrem alterações fisiológicas com potenciais efeitos deletérios que aumentam os riscos para uma variedade de doenças neurológicas,

cardiovasculares e musculoesqueléticas (MONTELEONE *et al.*, 2018). Isto porque receptores de estrogênio (ERα e ERβ) estão distribuídos em diversos tecidos, modulando fatores de transcrição, bem como interagindo com proteínas citoplasmáticas e nucleares envolvidas na transdução de sinais. Assim, a interação do estrógeno com proteínas citoplasmáticas e nucleares que participam de processos celulares fundamentais, incluindo, mas não somente, da biogênese, da dinâmica e da função mitocondrial, bem como da inflamação, promove proteção contra o desenvolvimento de diversas doenças(CHEN; BROWN; RUSSO, 2009; KLINGE, 2020; VILLA *et al.*, 2015).

No que se relaciona ao tecido muscular esquelético, em que ambos os receptores de estrogênio são expressos, estudos demostraram a participação do estrogênio na diferenciação e reparo celular, na homeostase metabólica e na função mitocondrial sendo considerado um regulador da massa e da função muscular (GALLUZZO *et al.*, 2009; HEVENER *et al.*, 2017; IKEDA; HORIE-INOUE; INOUE, 2019). De fato, estudos tem demostrado evidencias de que a deficiência hormonal decorrente da menopausa resulta em perda de força e massa muscular (TIIDUS; LOWE; BROWN, 2013). Em um estudo realizado com mulheres gêmeas monozigóticas na pós menopausa, no qual somente uma mulher de cada par utilizava reposição hormonal, foi demostrado que mulheres com reposição hormonal apresentam maior massa muscular e melhor desempenho em testes de força e potência muscular (PÖLLÄNEN *et al.*, 2010). Um outro estudo randomizado controlado demostrou também que mulheres que realizavam reposição hormonal apresentam maior quantidade de massa magra em relação àquelas que realizavam suplementação (PÖLLÄNEN *et al.*, 2010).

No entanto, a rica variabilidade de fatores vitais, tais como dieta, status socioeconômico, exposições ambientais e características genéticas dificultam a avaliação de variáveis específicas associadas à menopausa em humanos (KOEBELE; BIMONTE-NELSON, 2016). Assim, o uso de modelos animais, principalmente o modelo de ratas ovariectomizadas (OVX), tem sido útil para o esclarecimento das alterações musculares decorrentes da menopausa, bem como dos mecanismos subjacentes à tais alterações, uma vez que envolve uma população mais homogênea (KOEBELE; BIMONTE-NELSON, 2016). Ao encontro de estudos realizados em humanos, ratas OVX apresentam redução da área de secção transversa e da atividade contrátil muscular tão cedo quanto 8 a 14 semanas após a ovariectomia (DAGDEVIREN *et al.*, 2011; SUTHAM *et al.*, 2018; YWAZAKI *et al.*, 2016).

Embora os mecanismos por meio dos quais a perda dos hormônios ovarianos pode afetar negativamente o músculo esquelético sejam multifatoriais, uma vez que, os hormônios

ovarianos podem atuar em diversos sistemas no organismo, parece que o balanço inflamatório e a função mitocondrial intramuscular representam contribuintes centrais (COLLINS *et al.*, 2018; LIZCANO; GUZMÁN, 2014). A ovariectomia reduz a expressão de proteínas mitocondriais bem como a expressão de marcadores da sua biogênese, exercendo importante impacto na função mitocondrial, tal como evidenciado pelo aumento da produção de espécies reativas de oxigênio e pela redução da capacidade respiratória mitocondrial (BARBOSA *et al.*, 2016; CAPLLONCH-AMER *et al.*, 2014; CAVALCANTI-DE-ALBUQUERQUE *et al.*, 2014; SUTHAM *et al.*, 2018). Além disso, a ovariectomia altera as concentrações intramusculares de diversas citocinas. Por exemplo, foi observado, aumento da expressão de TNF-α no músculo esquelético, uma citocina pro-inflamatória que também foi associada a redução da função muscular após a ovariectomia (DAGDEVIREN *et al.*, 2011; KIM *et al.*, 2019), redução das concentrações de IL-10, uma citocina predominante anti-inflamatória, e aumento das concentrações de IL-6, uma citocina moduladora que está associada ao metabolismo energético, ao controle inflamatório e ao reparo muscular (KIM *et al.*, 2019).

Sabe-se que disfunções mitocondriais e a inflamação crônica podem induzir a perda de tecido muscular (BAUMANN *et al.*, 2016; GOMES *et al.*, 2017; LONDHE; GUTTRIDGE, 2015; MENG; YU, 2010). Em adição, é importante considerar que o balanço inflamatório e a função mitocondrial estão intimamente ligados. As disfunções mitocondriais promovem a perda do equilíbrio redox, o que pode resultar em dano celular e ativação de processos inflamatórios. Inversamente, citocinas pró-inflamatórias cronicamente ativadas, como o TNF-α, podem prejudicar a função e a biogênese mitocondrial, promovendo assim um ciclo vicioso pró-inflamatório (CHERRY; PIANTADOSI, 2015; VALERIO *et al.*, 2006).

A integridade muscular é essencial para prática de atividades de vida diária e ocupacionais, além da prática de atividade física, sendo assim importante para participação do indivíduo no contexto social, bem como para manutenção da saúde, com importante impacto na qualidade e expectativa de vida (CRUZ-JENTOFT; SAYER, 2019; SARTORI, R. ROMANELO, V. SANDRI, 2021). Desta forma, a busca por alternativas para prevenir a perda da integridade muscular em mulheres na pós-menopausa se torna uma questão de saúde pública, principalmente diante do aumento da expectativa de vida mundial, em que há um declínio na mortalidade tardia, e as mulheres passam a viver cerca de 1/3 de suas vidas no período pós-reprodutivo (KIRKWOOD, 2008; SARTORI, R. ROMANELO, V. SANDRI, 2021).

Um número crescente de evidências tem se concentrado em estratégias terapêuticas para prevenir os efeitos deletérios da falta de estrogênio em aspectos do músculo esquelético. Nesse

sentido, muitos estudos nesta área destacam os efeitos protetores da terapia de reposição estrogênica. No entanto, considerando que, de acordo com as diretrizes de manejo da menopausa as mulheres no climatério devem prevenir os efeitos deletérios relacionados à deficiência hormonal principalmente por meio de mudanças no estilo de vida, e considerando ainda, que é importante pesar os riscos cardiovasculares e de câncer associados a reposição hormonal individualmente (LOBO, 2017), observamos uma lacuna na literatura no que diz respeito ao treinamento de exercícios de resistência como estratégia terapêutica eficiente para prevenir as disfunções mitocondriais, assim como a terapia de reposição hormonal e, consequentemente, manter a integridade mitocondrial no músculo esquelético (CAPLLONCH-AMER *et al.*, 2014; CAVALCANTI-DE-ALBUQUERQUE *et al.*, 2014).

Assim, uma vez que o treinamento físico de resistência estimula a biogênese mitocondrial e melhora o estado redox no músculo em condições fisiológicas normais (EGAN; ZIERATH, 2013; LUNDBY; JACOBS, 2016), e que sendo o músculo esquelético um órgão endócrino secretor de miocinas com ações modulatórias locais e sistêmicas que podem contribuir para a homeostase muscular (PEDERSEN, 2011), acreditamos que o treinamento físico de resistência poderia prevenir o desequilíbrio redox e inflamatório induzido pela deficiência hormonal, mantendo o conteúdo mitocondrial do músculo da mesma forma que a terapia de reposição por estradiol. Por esse motivo, nosso estudo investigou o efeito do treinamento físico de resistência sobre a densidade mitocondrial, estado redox e marcadores inflamatórios em um modelo experimental de menopausa.

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2. ARTIGO CIENTÍFICO

Can endurance exercise training improve redox and inflammatory status and increase mitochondrial density in the skeletal muscle of ovariectomized rats?

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Abstract

Objective: To investigate the effect of moderate endurance training in skeletal muscle inflammatory biomarkers, mitochondrial density and aerobic capacity of ovariectomized rats.

Study design: Twelve weeks-old female Wistar rats were randomly assigned into two groups: Ovariectomized Aerobically Trained (OVX-AT, n=16) and Ovariectomized Not Aerobically Trained (OVX-NAT, n=16) rats. The training consisted of treadmill running at 50~70% of maximal running speed, one hour a day, 5 days/week during 8 weeks. Both groups performed a maximal treadmill test: (1) one day before, (2) in the end of the fourth and (3) after 24 hours in the follow up of 8 weeks. All rats were euthanized 48 hours after the last maximal treadmill test, the soleus muscle was harvested and processed for electron transmission microscopy and enzyme-linked immunosorbent assay (ELISA). Comparisons were performed by unpaired Student t-test or two-way ANOVA followed by Bonferroni post-hoc test. The significance level was set at 5%.

Main outcomes measures: Aerobic capacity (oxygen consumption, mechanical efficiency, time, speed and distance reached in a maximal exercise test), mitochondrial density and inflammatory biomarkers (IL-6, IL-10 and TNF-α).

Results: The OVX-AT presented improved aerobic capacity and increased soleus muscle mitochondrial density ($p<0.001$). Moreover OVX-AT exhibited increased levels of IL-6 and IL-10 ($p<0.05$) with no changes in TNF-α levels. Furthermore, the IL-10/TNF-α ratio was also higher in OVX-AT ($p<0.05$).

Conclusions: Endurance exercise training enhances skeletal muscle anti-inflammatory biomarkers and improve mitochondria density in OVX rats, factors that may contribute to prevent menopause deleterious effects on skeletal muscle integrity.

Keywords: Ovariectomy, endurance training, cytokines, mitochondria density, skeletal muscle.

1. Introduction

Postmenopausal increases the risk for metabolic disturbances and occurrence of musculoskeletal chronic disorders, e.g., osteoporosis, tendinopathies, and arthritis [1,2]. Taking into account skeletal muscle plays a central role in musculoskeletal disorders, maintenance of muscle integrity after menopause is essential to prevent future disabilities [1].

The mechanisms through which the ovarian hormone-deficiency negatively affect the muscle of postmenopausal women are multifactorial, highlighting the inflammatory aspects and mitochondrial dysfunction as major contributors [1,2]. Studies using ovariectomized (OVX) rats, i.e., the most common menopause animal model, reported decrease in mitochondrial biogenesis markers and respiratory capacity, and increase in skeletal muscle oxidative stress after ovariectomy [3–5]. In addition, ovariectomy alters the intramuscular levels of several cytokines. For example, ovariectomy enhance TNF-α expression, a pro-inflammatory cytokine that was also associated with reduced muscle function after ovariectomy [6,7], reduce IL-10 levels, a predominant anti-inflammatory cytokine, and increase IL-6 levels, a modulatory cytokine associated to energy metabolism, inflammatory control and muscle repair [7,8].

It is noteworthy that mitochondrial dysfunctions promote the redox imbalance, which induces cell damage and activation of inflammatory processes [9]. Additionally, a chronic stimulation of pro-inflammatory cytokines, e.g., tumour necrosis factor alpha (TNF-α), impairs mitochondrial function and biogenesis promoting a pro-inflammatory vicious cycle [9,10].

The growing number of evidences has focused on therapeutic strategies to prevent the deleterious effects of the lack of estrogen in aspects of skeletal muscle. In this sense, many studies highlights the mitochondrial protective effects of estrogen replacement therapy [4,5]. However, considering that postmenopausal women should primarily prevent hormone deficiency-related deleterious effects through lifestyle changes according to menopause management guidelines [11], there is a gap if regular endurance exercise training could be an efficient therapeutic strategy to prevent mitochondrial disfunction likewise hormone replacement therapy, and consequently maintain the mitochondrial integrity in the skeletal muscle [4,5].

In this context, taking into account that endurance exercise training stimulates mitochondrial biogenesis and improves redox and inflammatory status in muscle under normal physiological conditions [12–14], we believe that endurance exercise training could prevent the redox and inflammatory imbalance induced by hormone deficiency, maintaining the mitochondrial content in muscle likewise the estradiol replacement therapy. For this reason, our study investigated the endurance training effect on mitochondrial density, redox and inflammatory markers in an experimental model of menopause.

2. Methods

2.1. Animals

12-week-old females Wistar rats (n =30, mass=216±2.54g) were provided by the bioterism center of the Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. The animals were kept in a temperature-controlled room (22◦C), with a 12h dark:light cycle, and received standard chow and water *ad libitum*. All rats were treated similarly in terms of daily manipulation. All surgical procedures and protocols used were approved by Animal Use Ethics Committee of the Universidade Federal dos Vales do Jequitinhonha e Mucuri (protocol nº015/2019) and conducted in accordance with National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals.

2.2 Experimental design and sampling

The rats were randomly assigned into three groups: 1) Ovariectomized not aerobically trained rats (OVX-NAT, n=10), 2) Ovariectomized rats with estrogen replacement (OVX-ER, n=10) and 3), Ovariectomized aerobically trained rats (OVX-AT, n=10). All animals were submitted to ovariectomy and received a subcutaneously implanted Silastic® capsule containing 17β-estradiol (OVX-ER group) or vehicle (OVX-NAT and OVX-AT groups). Two weeks after ovariectomy, OVX-AT rats were submitted to an aerobic training protocol for 8 weeks. The OVX-NAT and OVX-ER groups were equally handled and placed near the treadmill for the same period to match OVX-AT group conditions such as noise and environment exposure. All animals were submitted to exercise familiarization and to the maximal effort treadmill test before and after the aerobic training protocol.

The rats were euthanized by decapitation. Both right and left soleus muscles were harvest, washed in ice-cold PBS (0.15M, pH 7.34), frozen in liquid nitrogen, and stored at -80ºC. The left soleus muscles were processed for oxidative stress evaluation and the right ones for inflammatory analyses. In addition, posterior mid belly fragments of the right soleus from three animals per group were dissected and chemically fixed for mitochondrial density assessment by transmission electron microscopy.

2.3 Ovariectomy

The animals were anesthetized (Ketamine 80 mg/kg + Xylazine 12 mg/kg), both lateral abdominal walls were trichotomized and an incision was made. The ovaries were located, the oviduct was sectioned to remove the ovaries and the incisions were stitched [3,4].

The animals received one dose of antibiotics (Pentabiotic, 24.000 UI/Kg) immediately after surgery, and two doses of analgesic (Flunixin meglumine, 2.5 mg/kg), immediately and 24h post-surgery. Animals had two weeks to recover from surgery before aerobic capacity evaluation. All rats recovered successfully.

2.4 Estrogen replacement

Immediately after ovariectomy, all animals received a subcutaneously implanted Silastic® capsule containing 360 μg of 17β-estradiol/mL in corn oil or vehicle (corn oil). The Silastic® capsules were made of 20-mm segments of Silastic® tubing (inner/outer diameter: 1.02/2.16 mm). To implant the capsule an incision was made in the rat dorsal region (10 mm), and the Silastic® capsule was inserted using forceps [15]. The incision was subsequently stitched. Silastic ® capsules were re-implanted after 5 weeks in order to maintain concentrations within the physiological range [15,16]. The effectiveness of estradiol replacement was confirmed by uterus final mass comparisons among groups [15,17].

2.5 Maximal effort exercise test

All animals were adapted to the treadmill (0.3 km/h, 10 min/day, 5 days) (Panlab, Havard Apparatus, Spain) [18,19]. This procedure intended to teach the animals which way to run and to identify rats unable to run for no specific reason. In this study, all rats ran successfully.

The maximal effort exercise test consisted of 0.18 km/h increments every 3 minutes, until the rat could no longer keep pace [18–20]. The purpose was to evaluate aerobic capacity and determine exercise training intensity. Oxygen consumption during the maximal exercise test (VO_{2max}) was performed by an indirect calorimeter (Panlab, Harvard Apparatus, Spain) coupled to the treadmill (airflow = 1.0 L/min) where the rats performed the test. VO_{2max} was measured continuously by a computerized system (Metabolism, Panlab, Harvard Apparatus, Spain) [18].

2.6 Endurance exercise training

The OVX-AT group performed exercise on a motor treadmill (Insight®, SP, Ribeirão Preto, Brazil) at low-moderate intensity (~50–70% maximal running speed) for one hour/day, 5 days/week for 8 weeks (total of 40 sessions), with a gradual increase in speed from 0.7 to 1.2 km/h [18,20].

2.7 Electron microscopy

Fragments of the soleus muscle were fixed in Karnovsky's solution (2.5% glutaraldehyde and 2% paraformaldehyde) in 0.1M cacodylate buffer pH 7.4 overnight at 4°C. Then, samples were post-fixed in a mixture of 2% (w/v) osmium tetroxide and 1.5% (w/v) potassium ferrocyanide for a minimum of 2 hours to enhance the contrast of organelles. Following, samples were washed in distilled water and kept in 2% uranyl acetate (*en bloc* staining) overnight, serially dehydrated in graded ethanol baths, and embedded in Epon 812. Finally, 50nm ultrathin sections were stained with Reynolds lead citrate. Transmission electron microscopy (TEM) was performed using a FEI Tecnai G2-12 Spirit at 80 kV. The images were acquired in a SIS-MegaView 3 CCD camera with 1376 x 1070 pixels. Twenty-four electron micrographs per animal were taken at a ×11.000 magnification. Images were randomly selected from central parts of muscle fibers and were analysed with ImageJ. Volume densities (Vv) of mitochondria were determined with the classic point counting method using a 252-point-grid (500 x 500 nm grid) projected onto each image [21,22].

2.8 Redox status and antioxidant enzyme activities

The Bradford method using bovine serum albumin was used as a standard to determine the sample's protein levels [23]. The reaction of the thiobarbituric acid with malondialdehyde was used to determine lipid peroxidation by thiobarbituric acid reactive substances (TBARS) levels [24]. The ferric reducing ability of plasma (FRAP), i.e., the reduction of ferric-tripyridyltriazine [Fe(III)-TPTZ] complex to ferrous-tripyridyltriazine [Fe(II)-TPTZ] was used to determine the total antioxidant capacity [25] . The quantification of superoxide dismutase activity (SOD) was based on the inhibition of the reaction between $O2^{\cdot -}$ and pyrogallol [26]. Catalase activity (CAT) was determined by measuring the decrease in H_2O_2 absorbance at 240 nm [27].

2.9 Inflammatory biomarkers

Soleus muscle samples were defrosted gradually from -80 to 4°C. Thereafter it was homogenized in extraction solution (1ml/ muscle 100g) containing PBS 01x (125mL), NaCl (2.925g), BSA (0.625g), EDTA (46,5mg), PMSF (2.125mg), benzethonium chloride (5,6mg), Tween 20 (62.5µL), aprotinin (2.5µ). The homogenate was then centrifugated at 10.000xg for 10min at 4°C. The supernatant was separated and used for analyses of IL-6, IL-10, and TNF-α

according to the manufacturer's instructions by ELISA kits (DuoSet, R&D Systems, United States).

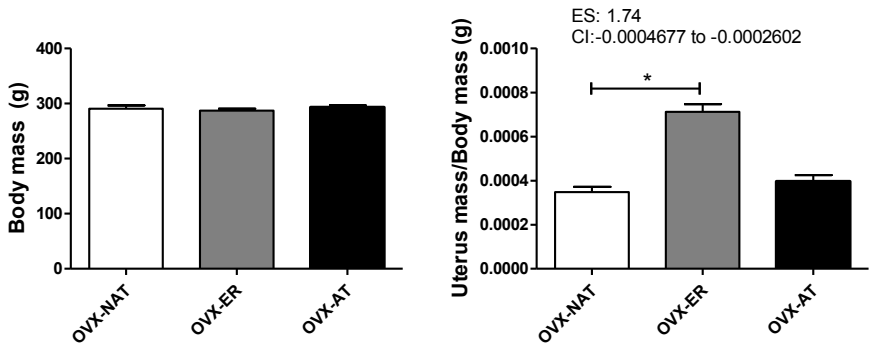
2.10 Statistical analyses

Data are reported as mean ± standard error (S.E.M.). Maximal exercise test comparisons were performed by ANOVA (two-way) followed by Dunnet post-hoc test. ANOVA one way was used for mitochondrial density, redox status, antioxidative enzymes, and inflammatory biomarkers analyses. Confidence interval (CI) and effect size (ES) for each significant analysis are also shown. The correlation between variables was evaluated using the Pearson coefficient. The significance level for all tests was set at 5%. Statistical analyses were performed with GraphPad Prism 5.0 and Gpower 3.1.9.2.

3 Results

Thereafter 10 weeks of ovariectomy, final body mass did no differ between groups (OVX-NAT: 290.50±6.38g; OVX-E2: 287.0±3.59g; OVX-AT: 293.00±3.88g). However, estradiol replacement induced a higher uterus mass in the OVX-ER (0.00070±0.00003g) compared with both placebo groups, OVX-NAT (0.00030 ±0.00002g) and OVX-AT (0.00039±0.00002g) (Figure 1).

Figure 1. Body and uterus mass



Data are reported as mean ± S.E.M. 95% confidence interval (CI). Effect size (ES) *p<0.05 (OVX-AT vs OVX-ER). One-way ANOVA.

Endurance exercise training improved exercise capacity in OVX-AT group compared with OVX-NAT group as observed in all variable obtained from the treadmill running

maximum exercise test (Table 1). Besides, time decreased VO_{2max} within OVX-NAT and OVX-ER rats along with all the other maximum exercise test variables. Endurance exercise training prevented the decrease in VO_{2max}, and improved the remaining assessed parameters.

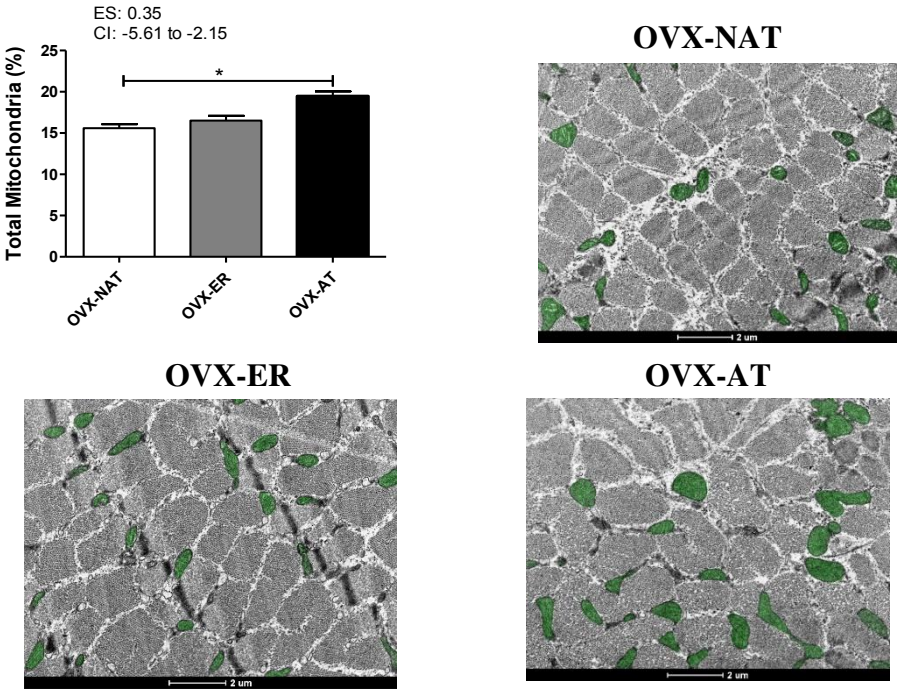
Table 1. Exercise capacity

Measurement	OVX-NAT (n=7)	OVX-ER (n=7)	OVX-AT (n=7)	CI (NAT vs ER)	CI (NAT vs AT)	ES	P[1]	P[2]	P[3]
$VO2_{max}$ (mL.kg-1.min-1)									
Initial	30.17 ± 0.51	30.78 ± 0.51	29.81 ± 0.76	-2.53 to 3.00	-3.76 to 1.78	1.73	<0.001	<0.001	0.055
Final	24.29 ± 0.62	24.18 ± 0.49	29.98 ± 1.12 *	-3.41 to 2.12	2.32 to 7.87				
Exercise efficiency (%)									
Initial	19.45 ± 0.90	19.01 ± 1.02	21.70 ± 1.42	-0.05 to 0.06	-0.02 to 0.08	0.95	<0.001	<0.001	<0.001
Final	20.69 ± 2.25	22.46 ± 2.26	37.51 ± 2.94 *	-0.04 to 0.06	0.09 to 0.20				
Distance (m)									
initial	239.10 ± 21.45	251.20 ± 24.66	263.30 ± 17.70	-90.97 to 121.70	-84.10 to 128.60	1.56	<0.001	0.079	<0.001
Final	136.80 ± 18.44	147.00 ± 23.03	530.40 ± 72.85 *	-112.80 to 99.85	262.60 to 475.30				
Time (s)									
initial	1215.00 ± 58.69	1246.00 ± 66.50	1836.00 ± 46.80	-195.90 to 268.70	-170.50 to 294.10	1.49	<0.001	0.635	<0.001
Final	888.60 ± 70.04	920.10 ± 82.74	1816.75 ± 136.0 *	-247.80 to 216.80	643.40 to 1108.00				
Speed (km/h)									
initial	1.31± 0.04	1.35 ± 0.06	1.35± 0.03	-0.20 to 0.27"	-0.20 to 0.27	1.41	<0.001	0.640	<0.001
Final	1.08± 0.06	1.06± 0.06	1.94 ± 0.09	-0.25 to 0.22"	0.62 to 1.10				

Data reported as mean ± S.E.M. 95% confidence interval (CI). Effect size (ES). p^1 interaction, p^2 time, p^3 treatment. *p <0.05 (OVX-AT vs OVX-NAT). ANOVA two-way.

The soleus muscle in OVX-AT group showed increased mitochondrial density ~20% (19.48±0.57%) compared with OVX-NAT (15.59 ± 0.47%), while OVX-ER (16.50±0.59%) showed no significant difference compared to the same group (Figure 2).

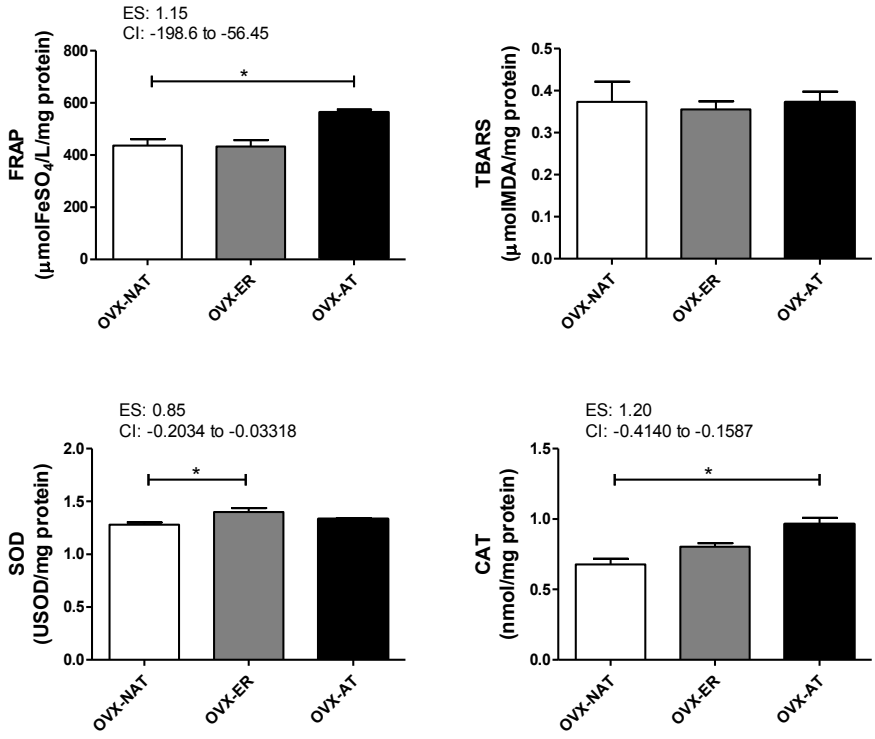
Figure 2. Mitochondrial density



Data are reported as mean ± S.E.M. 95% confidence interval (CI). Effect size (ES) * p<0.01. One-way ANOVA.

Regarding redox status, there was no significant changes in muscle TBARS levels among groups (OVX-NAT: 0.37±0.04mmolMDA/mg protein; OVX-ER: 0.35±0.01mmolMDA/mg protein; OVX-AT: 0.37±0.02mmolMDA/mg protein), and total antioxidative power (FRAP) levels were increased by endurance exercise training (OVX-NAT: 436.10±25.06 mmolFeSO4/L/mg protein; OVX-ER: 433.20 ± 23.94 mmolFeSO4/L/mg protein; OVX-AT: 563.60±10.80 mmolFeSO4/L/mg protein). Moreover, SOD levels increased in the OVX-ER rats (OVX-NAT: 1.28±0.01USOD/mg protein; OVX-ER: 1.40±0.03USOD/mg protein; OVX-AT: 1.33±0.00USOD/mg protein), while CAT levels increased in the OVX-AT group (OVX-NAT: 0.67±0.03nmol/mg protein; OVX-ER: 0.80±0.026 nmol/mg protein; OVX-AT: 0.96±0.04nmol/mg protein), both compared with control OVX-NAT.

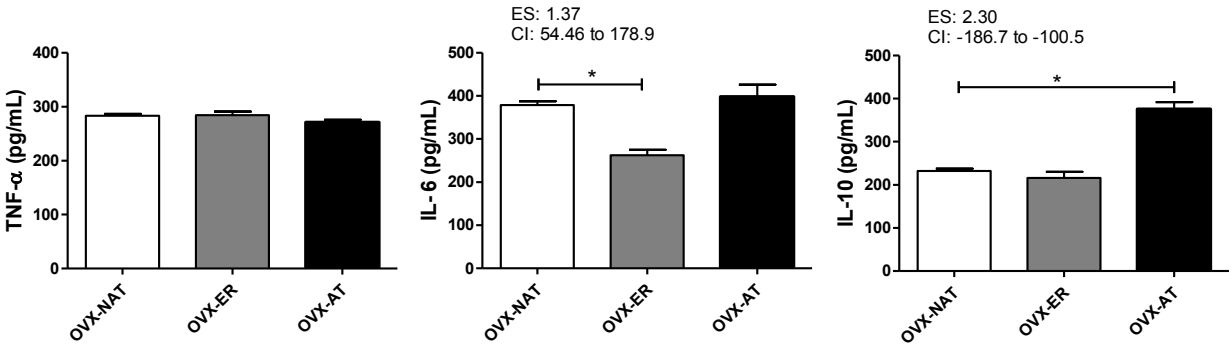
Figure 3. Redox status and anti-oxidative enzymes



Data are reported as mean ± S.E.M. 95% confidence interval (CI). Effect size (ES) * $p<0.01$. One-way ANOVA

TNF-α levels were unchanged after estradiol replacement or endurance exercise training (OVX-NAT: 283.4 ± 3.343 Pg/mg; OVX-ER: 284.4 ± 6.652 Pg/mg; OVX-AT: 271.5 ± 4.173 Pg/mg). However, while IL-6 levels reduced by 44% in the OVX-ER group (OVX-NAT: 378.9± 8.875 Pg/mg; OVX-ER: 262.3 ± 13.13 Pg/mg; OVX-AT: 398.5 ± 27.53 Pg/mg), IL-10 levels increased by 61% in the OVX-TA (OVX-NAT: 232.3 ± 5.249Pg/mg; OVX-ER: 216.0 ± 14.18 Pg/mg; OVX-AT: 375.9 ± 15.97 Pg/mg) (Figure 4).

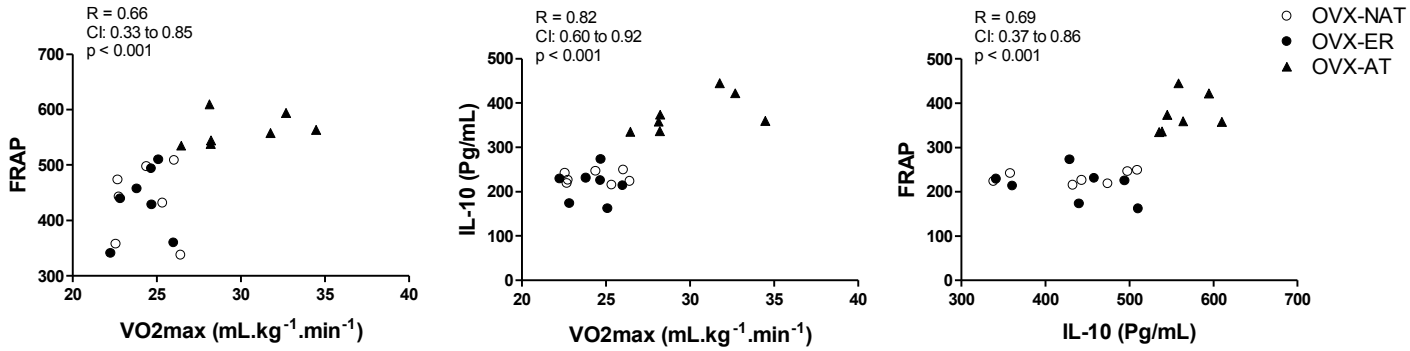
Figure 4. Inflammatory biomarkers



Data are reported as mean ± S.E.M. 95% confidence interval (CI). Effect size (ES) * p<0.01. One-way ANOVA.

Correlations analyses demonstrated moderate association between VO2max and FRAP, a strong association between VO2max and IL-10, and a moderate association between FRAP and IL-10 (Figure 5).

Figure 5. Correlations



Data are reported as mean ± S.E.M. Pearson correlation coefficient (R). 95% confidence interval (CI).

4 Discussion

Our study adds to the current literature that endurance exercise training, likewise hormone replacement therapy, prevents deleterious effects of ovarian hormone deprivation on aspects closely linked to skeletal muscle mass loss and physical performance. In this sense, endurance exercise training improved exercise capacity. This physical improvement was

accompanied by an enhancement of anti-oxidative and anti-inflammatory profile maintaining mitochondrial content in the skeletal muscle.

Studies demonstrated that ovariectomy reduces rats' performance during maximal exercise tests [19,28]. This deterioration in physical performance in OVX rats may have been induced by the decrease in the content and function mitochondrial [4,5]. In our study, aerobic capacity and mitochondrial density were effectively increased by endurance exercise training while estradiol replacement showed no effect on these parameters. To our knowledge, this is the first study that shows the effect of endurance training and estradiol on mitochondrial density of OVX rats by electron microscopy. This assay exhibits more accuracy in measurement of mitochondrial content compared with transcriptions factors levels, once that the increase of these markers does not necessarily means an increase in mitochondrial content. As mitochondrial adaptations are exercise-type sensitive, some studies indicate that the increase in mitochondrial density is induced by upregulation of mitochondrial lipid oxidative machinery and biogenesis [12,14]. Moreover, the proposed endurance exercise training protocol also improved exercise efficiency, which is an indicator of improved mitochondrial function [29].

It is noteworthy that ovariectomy may increase ROS production [3–5], which causes modifications in aspects related to the oxidative and inflammatory balance in favours of inflammation that lead to mitochondrial dysfunction [9,10]. In addition, our results indicate that both estradiol replacement therapy and endurance exercise training may protect muscle from oxidative stress in different ways. While estradiol replacement therapy increased SOD, endurance exercise training increased CAT levels. However, only endurance exercise training promoted a significant increase in total antioxidant capacity, as showed by the increase in FRAP levels.

Our data also demonstrated that estradiol reduced IL-6 level in the OVX-ER rats. The double physiological function of IL-6 has brought about much discussion in the scientific community. While some authors suggest IL-6 as an immune-modulatory cytokine that represents a marker of systemic low-grade inflammation in some chronic diseases and may participate in muscle waist signalling in specific conditions such as cachexia, others defend IL-6 to be especially beneficial for muscle metabolism and myogenesis [8]. Therefore, since the reduced levels of IL-6 caused by estradiol replacement therapy could contribute to a less inflammatory state, the intra muscle Il-6 expression and action must be further explored in future studies.

Despite IL-6 was unchanged by endurance exercise training, OVX-AT group presented higher levels of IL-10, an essentially anti-inflammatory myokine that downregulates pro-inflammatory signalling and protects the muscle against oxidative stress [9,10,30]. In fact, our data showed that IL-10 levels showed a moderate correlation to FRAP levels (R squared 0.69) while IL-6 showed no significant correlation to the same parameter (R squared 0.40). Additionally, we also observed a moderate correlation between FRAP and VO2max, and a strong correlation between IL-10 and VO2max, reinforcing the contribution of endurance exercise training to the total antioxidant capacity and anti-inflammatory status.

The probable mechanisms underlying the improvement in redox balance and anti-inflammatory profile maintaining the mitochondrial content induced by endurance exercise training in OVX rats must be further elucidated. As perspective, studies should investigate the long-term effects of endurance exercise training on musculoskeletal aspects compared to hormone replacement therapy in postmenopausal.

5 Conclusion

Endurance exercise training is more effective than estradiol replacement therapy to control aspects of the redox and inflammatory imbalance and to improve the skeletal muscle mitochondrial content. Thus, considering that endurance exercise training is more efficient to prevent skeletal muscle disorders and improve physical capacity, it should be considered as a first-line choice for the treatment of musculoskeletal disabilities in postmenopausal women.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Cândido Celso Coimbra: Resources

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3. CONCLUSÃO

O treinamento físico de endurance é mais eficaz do que a terapia de reposição de estradiol para controlar os aspectos do desequilíbrio redox e inflamatório e melhorar o conteúdo mitocondrial do músculo esquelético. Assim, considerando que o treinamento físico de endurance é mais eficiente na prevenção de disfunções musculares e melhora da capacidade física, deve ser considerado como uma escolha de primeira linha para o tratamento das deficiências musculoesqueléticas em mulheres na pós-menopausa.

ANEXOS I – NORMAS DA REVISTA

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ANEXO II – AUTORIZAÇÃO CEUA

C E R T I F I C A D O

Diamantina, 08 de maio de 2019.

Certificamos que a proposta intitulada " *Efeito do treinamento físico aeróbico na capacidade de exercício físico, biomarcadores inflamatórios e neurotróficos, estado redox e função mitocondrial em modelo animal que mimetiza menopausa*", registrada com o nº 015/2019, sob a responsabilidade de Ana Cristina rodrigues Lacerda e que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi **APROVADO** pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA/UFVJM) DA UNIVERSIDADE FEDERAL DOS VALES DO JEQUITINHONHA E MUCURI, em reunião de 08/05/2019.

Finalidade	( ) Ensino (X) Pesquisa Científica
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Sexo	Fêmea
Origem	Biotério do Centro Integrado de Pós-Graduação e Pesquisa em Saúde (CIPq) da UFVJM. Campus JK, Dimantina, MG.

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Flávio de Castro Magalhães
Coordenador da Comissão de Ética no Uso de Animais / UFVJM

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